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Discrete stages of human intrathymic differentiation: Analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage

(T-cell ontogeny/human thymus/hybridoma)

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ABSTRACT **A series of monoclonal antibodies was used to define three discrete stages of human intrathymic T-cell differentiation. The earliest stage was confined to <10% of thymocytes, which were reactive with both OKT9 and OKT10. Subsequently, approximately 70% of human thymocytes acquired a thymocyte-restricted antigen, OKT6, lost OKT9 antigen, and expressed reactivity with OKT4 and OKT5. These last two monoclonal antibodies were previously shown to define inducer (helper) and cytotoxic/suppressor populations, respectively, in peripheral blood. The OKT4$^+$, OKT5$^+$, OKT6$^+$ "common" thymocyte population represents the majority of thymocytes and accounts for more than 70% of thymocytes. With further maturation, thymocytes lose OKT6 reactivity, segregate into OKT4$^+$ and OKT5$^+$ subsets, and acquire reactivity with OKT3 (and OKT1). This latter stage corresponds to the more functionally mature subset. The possible relationship of acute lymphoblastic leukemia of T-cell lineage to these proposed stages of intrathymic differentiation was determined. Analysis of 25 tumor populations showed that 21 could be related to one or another differentiative stage. The majority (15/21) were derived from an early thymocyte or prothymocyte subpopulation, 5/25 were derived from a common thymocyte subpopulation, and 1/25 was derived from a mature (OKT3$^+$) subpopulation. These data suggest that is it now possible to define stages of T-cell differentiation that can be related to T-cell malignancies in humans.**

The importance of a thymic microenvironment in the differentiation and functional maturation of T cells has been demonstrated in several species. Moreover, profound changes in cell-surface antigens mark the various stages of T-cell ontogeny (1–7). For example, in the murine system, the first differentiative steps from stem cell to thymocyte are associated with acquisition of TL, Thy1, and Ly1,2,3 surface antigens. Within the thymus gland, the vast majority of murine lymphocytes are Ly1,2,3$^+$, TL$^+$, and Thy1$^+$. With further maturation, a subset of thymic lymphocytes becomes TL$^-$ and, concomitantly, has a diminution of Thy1 antigen and an increase in H2 antigen. This latter phenotype is characteristic of virtually all peripheral T cells. In addition, a significant number of Ly1,2,3$^+$ cells diverge, resulting in two subsets of T cells, Ly1$^+$ and Ly2,3$^+$. These subsets have been programmed for their respective inducer (helper) and cytotoxic/suppressor functions (8).

The precise stages of human intrathymic differentiation have been largely ill defined owing to the paucity of probes capable of correlating cell-surface antigenic changes with distinctive points of maturation. More recent studies using heteroantisera or monoclonal antibodies have demonstrated that human thymocytes express an antigen homologous to murine TL, termed HTL (9–11). Although present on thymocytes, HTL is not found on normal peripheral T cells. Thus, migration into the circulation is associated with loss of the thymic HTL antigen. In addition, HTL$^-$ peripheral T cells are reactive with OKT1 and, hence, are OKT1$^+$. The OKT1 monoclonal antibody is reactive with 100% of peripheral T cells and 10% of thymocytes (12). These thymocytes were the most functionally mature and probably ready for peripheral exportation. Human peripheral T cells also consist of functionally distinct subsets (13–19).

In the present study, we used a series of monoclonal antibodies reactive selectively with subpopulations of human thymocytes and inducer and suppressor T-cell subsets in order to further define stages of thymic differentiation (18, 19). We show that three major stages of intrathymic differentiation exist and that the majority of tumor populations from patients with acute lymphoblastic leukemia of T-cell lineage (T-ALL) can be shown to arise, in large part, from a single discrete stage of thymocyte development.

MATERIALS AND METHODS

Isolation of Lymphocyte Populations. Human peripheral blood mononuclear cells were isolated by Ficoll/Hypaque density centrifugation (Pharmacia). Subsequently, lymphocytes were separated into E rosette-positive (E$^+$) and E rosette-negative (E$^-$) subpopulations by use of 5% sheep erythrocytes (Microbiological Associates, Walkerville, MD) as described (15). Normal human thymus was obtained from patients 2 months to 12 years old who had had portions of their thymus removed during corrective cardiac surgery. Fresh thymic fragments were placed immediately in 5% fetal calf serum, finely minced with forceps, and made into single-cell suspensions by pressing through stainless steel mesh. The thymocytes so obtained were >95% viable and ≥90% E$^+$. All studies with thymic lymphocytes were performed in the fresh state. Human bone marrow cells were obtained with needle aspiration from the posterior iliac crest of normal volunteers.

Cell Lines of T Lineage and T-ALL Cells. T-cell lines CEM, HSB-2, and MOLT-4 were kindly donated by H. Lazarus (Sidney Farber Cancer Institute, Boston, MA). Leukemic cells were obtained from 25 patients with the diagnosis of T-ALL. These individual tumors had been determined to be of T-cell lineage by their spontaneous rosette formation with sheep erythrocytes (>20% E$^+$) and their reactivity with T-cell-specific heteroantisera anti-HTL (B.K.) and A99, as described (9, 20).

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Abbreviation: T-ALL, acute lymphoblastic leukemia of T-cell lineage.

Tumor populations were cryopreserved at −196°C vapor-phase liquid nitrogen with 10% dimethyl sulfoxide and 20% AB human serum until the time of surface characterization. All tumor populations analyzed were more than 90% blasts by Wright/Giemsa morphology of cytocentrifuge preparations.

Production and Characterization of Monoclonal Antibodies. Monoclonal antibodies OKT1, OKT3, OKT4, and OKT5 were produced and characterized as described (12, 18, 19, 21–23). In brief, these antibodies were shown to be restricted in their reactivity to cells of T lineage. OKT1 and OKT3 reacted with 100% of peripheral T cells and approximately 10% of thymocytes (12, 21, 22). The OKT1-reactive ($OKT1^+$) thymocyte population contained the functionally mature thymocyte population responsive to alloantigens in mixed lymphocyte culture. In contrast, OKT4 and OKT5 reacted with the majority of thymocytes and with 55% and 20%, respectively, of the peripheral T cells. Functionally, OKT4 defined the human inducer (helper) T-cell subset whereas OKT5 defined the human suppressor/cytotoxic population (18, 19, 21, 23). Monoclonal antibodies OKT6, OKT8, OKT9, and OKT10 were produced and characterized by described techniques after immunization of CAF_1 mice with human thymocytes (12). Hybridoma cultures containing antibody reacting predominantly with E^+ or thymocyte populations (or both) were selected, cloned, and recloned by limiting dilution methods in the presence of feeder cells. Malignant ascites were then developed and used for analysis. Table 1 provides a brief summary of the reactivity pattern of these monoclonal antibodies.

Cytofluorographic Analysis. Cytofluorographic analysis of all cell populations was performed by indirect immunofluorescence with fluorescein-conjugated goat anti-mouse IgG (Meloy Laboratories, Springfield, VA) on a Cytofluorograf FC200/4800A (Ortho Instruments, Westwood, MA) as described (12). In experiments involving antibody- and complement-mediated lympholysis, thymocytes and peripheral T cells were cultured overnight after selective lysis and then subsequently analyzed on the Cytofluorograf.

Lysis of Lymphoid Populations with Monoclonal Antibody and Complement. Forty × 10^6 peripheral T cells or thymocytes were placed in a 15-ml plastic tube (Falcon). Cell pellets were resuspended in 0.8 ml of OKT3, OKT4, OKT8, or normal ascites control diluted 1:200 in phosphate-buffered saline and incubated at 20°C for 60 min. Subsequently, 0.2 ml of fresh rabbit complement was added to the antibody-treated populations, which were further incubated at 37°C in a shaking water bath for 60 min. At the end of this time, cells were spun down and viable cells were enumerated by Trypan blue exclusion. After counting, cells were washed two additional times in 5% fetal calf serum, placed in final medium [RPMI 1640 (GIBCO) containing 20% AB^+ human serum, 1% penicillin/streptomycin, 200 mM L-glutamine, 25 mM Hepes, and 0.5% sodium bicarbonate], and incubated overnight in a humid atmosphere with 5% CO_2 at 37°C.

Table 1. Reactivity of monoclonal antibodies on human lymphoid populations*

Antibody	Peripheral blood (30)† E^+	Peripheral blood (30)† E^-	Bone marrow (6)†	Thymus (22)†
OKT6	0	0	0	70
OKT8	30	0	<2	80
OKT9	0	0	0	≤10
OKT10	<5	10	≤20	95

* Percent reactive cells is given and represents the mean reactivity.
† Numbers in parentheses represent the number of samples tested.

RESULTS

Characterization of Reactivity of Monoclonal Antibodies on Normal Human Thymocytes. Fig. 1 shows a representative fluorescence pattern obtained on the Cytofluorograf after thymocyte suspensions reacted with a 1:500 dilution of OKT3, OKT4, OKT5, OKT6, OKT8, OKT9, OKT10, and fluorescein-conjugated goat anti-mouse IgG. Similar patterns of reactivity were seen with 12 additional thymocyte populations tested. As shown, significant differences exist in both the percentage of reactivity and fluorescence intensity with each of these monoclonal antibodies. For example, OKT9 reacted with approximately 10% of thymocytes with low fluorescence intensity whereas OKT5, OKT6, OKT8, and OKT10 reacted with approximately 70–95% of thymocytes at a higher fluorescence intensity. OKT4, which reacted with 75% of thymocytes, was intermediate between OKT9 and the monoclonal antibodies that gave a pattern of greater fluorescence intensity. In addition, Fig. 1 shows that approximately 15% of thymocytes were detected with OKT3 by indirect immunofluorescence. Not shown is OKT1, whose pattern of reactivity was virtually identical with that of OKT3 on thymocytes.

Cell-Surface Antigen Distribution on Thymocytes and Peripheral T Cells. In prior studies, it was shown that the antigen defined by OKT4 was restricted to the human peripheral T-cell inducer (helper) population whereas that defined by OKT5 was restricted to the human cytotoxic/suppressor population. Moreover, in peripheral blood, these antigens were represented on discrete, nonoverlapping subsets (18, 19). The observation that, by indirect immunofluorescence, the vast majority of human thymocytes were both reactive with OKT4 and OKT5 suggested that a sizeable portion of thymocytes possessed both antigens.

To determine whether the antigens defined by OKT4 or OKT5 were on the same population or on different populations as is found in peripheral blood T cells, we performed a series of lysis studies. Because only OKT3, OKT4, and OKT8 were complement-fixing monoclonal antibodies, these three were used. As shown in Table 2, the entire peripheral T-cell population reacted with OKT3 whereas OKT4, OKT5, and OKT8 reacted with 60%, 25%, and 34% of T cells, respectively. Lysis with OKT4 and complement left only 38% of the cells; thus, it diminished the total number by 62% and left no remaining $OKT4^+$ cells. In addition, the percentage of $OKT5^+$ and $OKT8^+$ cells increased and there was no effect on the absolute

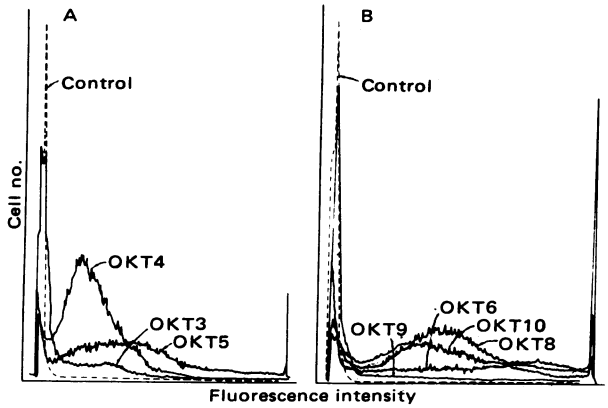

FIG. 1. Immunofluorescence profile of normal human thymocytes with monoclonal antibodies. As shown, both the fluorescence intensity and percentage of reactivity on thymocytes vary with the specific antibody tested (1:500 dilution). Background fluorescence staining was obtained by incubating each population with a 1:500 dilution of ascitic fluid from a mouse injected with a nonproducing hybrid clone.

Table 2. Differences in distribution of antigens defined by monoclonal antibody on human peripheral T cells and thymocytes

Lymphoid population	Total cells recovered $\times 10^{-6}$	% reactivity of residual cells with monoclonal antibodies: OKT3	OKT4	OKT5	OKT6	OKT8	OKT9	OKT10
Peripheral T cells								
Untreated*	40 —	98	60	25	0	34	—	—
OKT4 + C′	15.2 (38%)	95	0	70	0	95	—	—
OKT8 + C′	25.2 (63%)	95	92	0	0	0	—	—
Thymocytes								
Untreated*	40 —	30	75	70	65	75	8	92
OKT4 + C′	10 (25%)	80	0	85	10	55	6	75
OKT8 + C′	9.5 (24%)	85	65	0	13	0	5	85
OKT3 + C′	30 (75%)	0	60	80	82	90	12	86

* Untreated populations and populations treated with complement (C′) alone were indistinguishable on re-analysis. Nonspecific lysis was ≤5% in all cases. Results are representative of six experiments.

number of OKT5$^+$ and OKT8$^+$ T cells. These studies suggested that OKT4$^+$ was distinct from the OKT5$^+$ and OKT8$^+$ populations. Further support for this conclusion was obtained by lysis of peripheral T cells with OKT8 and complement. In this case, the percentage of OKT4$^+$ T cells increased, the absolute number remained the same, and OKT8$^+$ and OKT5$^+$ populations were eliminated. Moreover, these results demonstrated that the OKT8$^+$ population was reciprocal to the OKT4$^+$ population and contained the entire OKT5$^+$ T-cell subset.

Studies similar to those described above were performed with human thymocyte populations; these gave different results (Table 2). After lysis with either OKT4 or OKT8, only 25% of thymocytes remained; thus, approximately 75% of thymocytes were OKT4$^+$ or OKT8$^+$. The majority of residual thymocytes were reactive with OKT3 whereas only a minority was reactive with OKT6. These findings demonstrate that a major population of human thymocytes bear the OKT4, OKT5, OKT6, and OKT8 surface antigens on the same cell. In addition, Table 2 demonstrates that after treatment with OKT8 or OKT4, there is a marked increase in the mature thymocytes bearing the OKT3 antigen. Thus, the majority of OKT3-reactive thymocytes have already segregated into OKT4$^+$ or OKT8$^+$ subsets because the major proportion of residual cells after OKT4 or OKT8 lysis are OKT3$^+$. If the OKT3$^+$ subpopulation were both OKT4$^+$ and OKT8$^+$, then lysis with either monoclonal antibody should have removed the OKT3-reactive thymocytes.

To further determine the relationship of OKT3-reactive thymocyte subpopulations to the other monoclonal-antibody-defined thymocyte fractions, we treated thymocytes with OKT3 and complement and compared the residual cells to untreated thymocyte populations. As shown in Table 2, OKT3 and complement removed 25% of thymocytes. Moreover, there was no major loss of OKT4-, OKT5-, OKT6-, or OKT8-reactive populations. These findings suggest that the vast majority of thymocytes bearing the OKT6 marker are contained in the OKT3$^-$ population. In addition, they further suggest that thymocytes simultaneously expressing antigens defined by OKT4, OKT5, and OKT8 are likewise restricted to the OKT3$^-$ population. The OKT9-reactive population of thymocytes was not diminished after treatment of the unfractionated thymocytes with OKT3 and complement, thus showing that the OKT9$^+$ subpopulation is largely restricted to the OKT3$^-$ thymocyte population.

Taken together, the above studies suggest that three major stages of thymic differentiation exist in humans. As shown in Fig. 2, virtually all thymocytes bear the OKT10 marker. In addition, thymocytes acquire at an early stage the OKT9 marker (Thy1 and Thy2, respectively). This stage defines the minority of thymocytes and accounts for approximately 10% of the unfractionated population. Subsequently, human thymocytes acquire a thymocyte-distinctive antigen defined by OKT6 and concurrently express OKT4, OKT5, and OKT8 (Thy4). This latter subpopulation represents the majority of thymocytes and accounts for more than 70% of the thymic population. With further maturation, thymocytes lose OKT6 reactivity, acquire OKT3 (and OKT1) reactivity, and segregate into OKT4$^+$ (Thy7) and OKT5$^+$/OKT8$^+$ (Thy8) subsets. Last, it appears that as the thymocyte is exported into the peripheral T-cell compartment, it loses the OKT10 marker because this antigen is lacking on virtually all peripheral T lymphocytes. Possible transitional states between these three major stages of thymic development are designated by Thy3, Thy5, and Thy6 in Fig. 2.

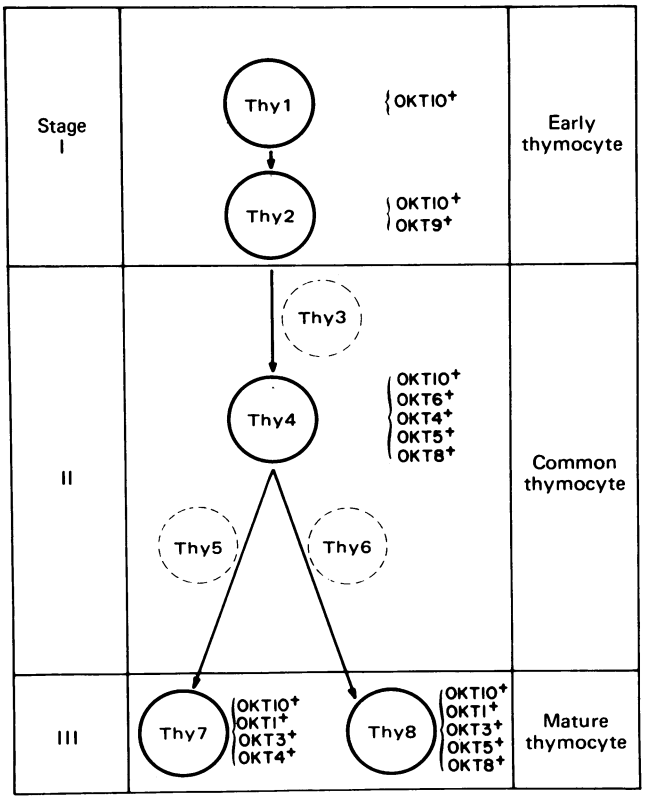

FIG. 2. Stages of intrathymic differentiation in humans. Three discrete stages of thymic differentiation can be defined on the basis of reactivity with monoclonal antibodies by use of indirect immunofluorescence. Solid circles represent thymocytes within specific stages of defined phenotype; broken circles represent hypothetical transition states between these stages.

Table 3. Cell-surface characteristics of T-ALL

Differentiative status*	Reactivity with monoclonal antibodies:†							No. of T-ALL tested (n = 25)
	OKT3	OKT4	OKT5	OKT6	OKT8	OKT9	OKT10	
Stage I								
A. Prothymocyte (Thy1)	−	−	−	−	−	−	+	7
B. Early thymocyte (Thy2)	−	−	−	−	−	+	+	8
Stage II								
Common thymocyte								
(Thy3)	−	+	−	+	+	+	+	1
(Thy4)	−	+	−	+	+	−	+	1
(Thy3 → 4)	−	−	−	+	−	−	+	2
(Thy4 → 6)	−	−	−	+	+	−	+	1
Stage III								
Late thymocyte (Thy8)	+	−	+	−	+	−	+	1
								Total 21‡

* Thy designation refers to Fig. 2.

† Positive (+) reactivity was defined as ≥30% specific fluorescence above background control; negative (−) reactivity was indistinguishable from background staining on tumor-cell suspensions.

‡ An additional four tumors could not be easily categorized into stages I–III. See text for details of their characterization.

Surface Antigens Expressed by Leukemic Blasts of T Lineage. Because T-ALL is thought to be derived from immature thymocytes, it seemed important to determine whether tumor cells from individuals with T-ALL were related to any of these proposed stages of intrathymic differentiation (20). Consequently, 25 tumor-cell populations from individuals with T-ALL and three T-cell lines that had been studied with conventional anti-T cell reagents and E rosetting were investigated (19). As shown in Table 3, the majority of T-ALL cells were reactive with either OKT10 alone or OKT9 and OKT10 and failed to react with the other monoclonal antibodies. Thus, 15 of 25 tumors studied appeared to possess early thymocyte antigens (stage I). In contrast, 5 of 25 tumors were reactive with OKT6, suggesting derivation from a more mature thymus population (stage II). This T-ALL group was itself heterogeneous with respect to OKT4, OKT8, and OKT9 reactivity, as shown in Table 3. Cells from two of five patients possessed most of the common thymocyte antigens, including OKT4, OKT6, and OKT8. OKT5 was not present on any of these five stage II tumors even though OKT8 reactivity was observed. This latter result clearly suggests that OKT5 and OKT8 define different antigens. Finally, 1 of 25 patients' tumors came from a mature thymocyte population (stage III) as defined by its reactivity with OKT3. This individual's tumor was additionally reactive with OKT5, OKT8, and OKT10. Of the 25 leukemic populations analyzed, only four tumors could not be clearly categorized. Three were positive with OKT4 and OKT8, but lacked OKT3 and OKT6, and most likely represented transitions from Thy4 to Thy7,8. One of 25 tumors appeared to be a transition from Thy3 to Thy4 because it possessed OKT8 and OKT10 reactivity.

T-cell lines derived from T-ALL tumor populations also represented cells from a specific state of intrathymic differentiation. As shown in Table 4, line HSB-2 was reactive with OKT9 and OKT10 exclusively and would therefore define a tumor population derived from stage I. In contrast, line CEM was reactive with OKT4, OKT6, OKT8, OKT9, and OKT10 and appeared to be derived from a stage II thymocyte. Finally, line MOLT-4 seems to represent a leukemic transformation at a stage between HSB-2 and CEM because it expressed OKT6, OKT8, OKT9, and OKT10.

Table 4. Reactivity with monoclonal antibodies*

Cell line	OKT3	OKT4	OKT5	OKT6	OKT8	OKT9	OKT10
HSB-2	−	−	−	−	−	+	+
CEM	−	+	−	+	+	+	+
MOLT-4	−	−	−	+	+	+	+

* Criteria for − and + reactivity were the same as in Table 3.

DISCUSSION

In the present study, the distribution of cell-surface antigens expressed on human thymocytes was compared with that of peripheral T cells by using monoclonal antibodies as probes. Major differences were found in the cell-surface antigen composition of thymocytes and peripheral T lymphocytes. For example, approximately 65–70% of human thymocytes reacted with OKT6 whereas peripheral lymphoid cells were unreactive with OKT6. In addition, OKT10 was expressed on >95% of thymocytes and <5% of peripheral T cells. Because the antigen defined by OKT10 was also found on a significant number of bone marrow cells as well, it appeared that this antigen was not restricted to cells of T lineage but that it was rather an early differentiation antigen. OKT9 was found on approximately 10% of thymocytes but was lacking on bone marrow or more mature cells. OKT1 and OKT3, in contrast, defined antigens present on all peripheral T cells but expressed on only a small fraction of thymocytes which were previously shown to be more mature functionally (12).

Prior studies demonstrated that OKT4 and OKT5 were present on discrete, nonoverlapping subpopulations of peripheral T lymphocytes as determined by indirect immunofluorescence (18, 19). In the present study, lysis of peripheral T cells with OKT4 and complement reaffirmed these findings. Moreover, it showed that the $OKT8^+$ population contains the entire $OKT5^+$ subpopulation of cells and is reciprocal to the $OKT4^+$ subset. In contrast to peripheral T cells, 70–80% of thymocytes express both OKT4 and OKT5. Lysis studies provided direct evidence for this and showed that, in addition to OKT4 and OKT5, OKT6 and OKT8 are expressed on the same population of cells. Moreover, lysis with OKT3 did not diminish the number of OKT6- or OKT9-reactive cells, suggesting that these antigens correlated with discrete stages of earlier differentiation. Because lysis studies with OKT4 or OKT8 enriched the OKT3-reactive population and did not eliminate OKT8 or OKT4, respectively, it would appear that the OKT4 and OKT8 antigens have already diverged in the more mature $OKT3^+$ population.

These studies suggest that three major compartments of thymic differentiation exist in humans (Fig. 2): (I) thymocytes bearing OKT9 or OKT9 and OKT10; (II) thymocytes bearing OKT4, OKT5, OKT6, and OKT8; and (III) thymocytes bearing OKT1 and OKT3 (and OKT4 or OKT5/OKT8). Analysis of tumor populations in patients with T-ALL and in T-cell lines derived from patients with T-ALL demonstrated that these lymphoid malignancies were thymocyte derived and, more importantly, restricted to discrete stages of differentiation. A majority of human T-ALL was shown to be derived from an early thymocyte or possibly even prothymocyte compartment (stage I). In contrast, only 20% of the T-ALL populations analyzed were derived from the stage II thymocyte population, which numerically represents the vast majority of normal human thymocytes. One of 25 patients had a tumor population derived from the most mature fraction of the thymus (stage III). In addition, four patients' leukemic cells were less well defined and could represent either transition states between these more discrete stages of differentiation or perhaps some abnormality of differentiation associated with malignancy.

In earlier studies with TH_2 heteroantisera, approximately 20% of T-ALL tumor populations were TH_2^+ (20). These tumor populations were precisely the ones reactive with monoclonal antibodies defining stage II thymocytes. In that prior study, it was shown that patients with TH_2^+ T-ALL tended to have more prolonged disease-free survival than their TH_2^- T-ALL counterparts. This would appear to correlate with the more mature state of differentiation of those tumor cells.

The present study shows that it is possible to define distinct intrathymic stages of differentiation and to relate T-cell leukemias in humans to these stages. Because functional activity is acquired at the level of the OKT1, $OKT3^+$ thymocyte subpopulation, it is possible that those T-ALLs with functional activity will be derived from this mature population of cells (12). In addition to providing a potentially important set of reagents for investigating thymic maturation, the present study allows for the orderly categorization of T-cell malignancies.

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